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Method 1008.0: Sea Urchin, *Arbacia punctulata,* Fertilization Test; Chronic Toxicity

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Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms

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SECTION 15

TEST METHOD

SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST METHOD 1008.0

15.1 SCOPE AND APPLICATION

15.1.1 This method, adapted in part from USEPA (1987e), measures the toxicity of effluents and receiving water to the gametes of the sea urchin, *Arbacia punctulata*, during a 1 h and 20 min exposure. The purpose of the sperm cell toxicity test is to determine the concentration of a test substance that reduces fertilization of exposed gametes relative to that of the control.

15.1.2 Detection limits of the toxicity of an effluent or chemical substance are organism dependent.

15.1.3 Brief excursions in toxicity may not be detected using 24-h composite samples. Also, because of the long sample collection period involved in composite sampling and because the test chambers are not sealed, highly volatile and highly degradable toxicants in the source may not be detected in the test.

15.1.4 This test is commonly used in one of two forms: (1) a definitive test, consisting of a minimum of five effluent concentrations and a control, and (2) a receiving water test(s), consisting of one or more receiving water concentrations and a control.

15.2 SUMMARY OF METHOD

15.2.1 The method consists of exposing dilute sperm suspensions to effluents or receiving waters for 1 h. Eggs are then added to the sperm suspensions. Twenty minutes after the eggs are added, the test is terminated by the addition of preservative. The percent fertilization is determined by microscopic examination of an aliquot from each treatment. The test results are reported as the concentration of the test substance which causes a statistically significant reduction in fertilization.

15.3 INTERFERENCES

15.3.1 Toxic substances may be introduced by contaminants in dilution water, glassware, sample hardware, and testing equipment (see Section 5, Facilities, Equipment, and Supplies).

15.3.2 Improper effluent sampling and handling may adversely affect test results (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.4 SAFETY

15.4.1 See Section 3, Health and Safety.

15.5. APPARATUS AND EQUIPMENT

15.5.1 Facilities for holding and acclimating test organisms.

15.5.2 Laboratory sea urchins, *Arbacia punctulata*, culture unit -- See Subsection 15.6.19, culturing methods below and Section 4, Quality Assurance. To test effluent or receiving water toxicity, sufficient eggs and sperm must be available.

15.5.3 Samplers -- automatic sampler, preferably with sample cooling capability, that can collect a 24-h composite sample of 5 L.

15.5.4 Environmental chamber or equivalent facility with temperature control (20 ± 1°C).

15.5.5 Water purification system -- Millipore Milli-Q®, deionized water (DI) or equivalent.

15.5.6 Balance -- Analytical, capable of accurately weighing to 0.00001 g.

15.5.7 Reference weights, Class S -- for checking performance of balance. Weights should bracket the expected weights of materials to be weighed.

15.5.8 Air pump -- for oil-free air supply.

15.5.9 Air lines, and air stones -- for aerating water containing adults, or for supplying air to test solutions with low DO.

15.5.10 Vacuum suction device -- for washing eggs.

15.5.11 Meters, pH and DO -- for routine physical and chemical measurements.

15.5.12 Standard or micro-Winkler apparatus -- for determining DO (optional).

15.5.13 Transformer, 10-12 Volt, with steel electrodes -- for stimulating release of eggs and sperm.

15.5.14 Centrifuge, bench-top, slant-head, variable speed -- for washing eggs.

15.5.15 Fume hood -- to protect the analyst from formaldehyde fumes.

15.5.16 Dissecting microscope -- for counting diluted egg stock.

15.5.17 Compound microscope -- for examining and counting sperm cells and fertilized eggs.

15.5.18 Sedgwick-Rafter counting chamber -- for counting egg stock and examining fertilized eggs.

15.5.19 Hemacytometer, Neubauer -- for counting sperm.

15.5.20 Count register, 2-place -- for recording sperm and egg counts.

15.5.21 Refractometer -- for determining salinity.

15.5.22 Thermometers, glass or electronic, laboratory grade -- for measuring water temperatures.

15.5.23 Thermometers, bulb-thermograph or electronic-chart type -- for continuously recording temperature.

15.5.24 Thermometer, National Bureau of Standards Certified (see USEPA Method 170.1, USEPA, 1979b) -- to calibrate laboratory thermometers.

15.5.25 Ice bucket, covered -- for maintaining live sperm.

15.5.26 Centrifuge tubes, conical -- for washing eggs.

15.5.27 Cylindrical glass vessel, 8-cm diameter -- for maintaining dispersed egg suspension.

15.5.28 Beakers -- six Class A, borosilicate glass or non-toxic plasticware, 1000 mL for making test solutions.

15.5.29 Glass dishes, flat bottomed, 20-cm diameter -- for holding urchins during gamete collection.

15.5.3 Wash bottles -- for deionized water, for rinsing small glassware and instrument electrodes and probes.

15.5.31 Volumetric flasks and graduated cylinders -- Class A, borosilicate glass or non-toxic plastic labware, 10-1000 mL for making test solutions.

15.5.32 Syringes, 1-mL, and 10-mL, with 18 gauge, blunt-tipped needles (tips cut off) -- for collecting sperm and eggs.

15.5.33 Pipets, volumetric -- Class A, 1-100 mL.

15.5.34 Pipets, automatic -- adjustable 1-100 mL.

15.5.35 Pipets, serological -- 1-10 mL, graduated.

15.6.36 Pipet bulbs and fillers -- PROPIPET®, or equivalent.

15.6 REAGENTS AND CONSUMABLE MATERIALS

15.6.1 Sea Urchins, *Arbacia punctulata* minimum 12 of each sex.

15.6.2 Food -- kelp, *Laminaria* sp., or romaine lettuce for the sea urchin, *Arbacia punctulata*.

15.6.3 Standard salt water aquarium or Instant Ocean Aquarium (capable of maintaining seawater at 15°C) -- with appropriate filtration and aeration system.

15.6.4 Sample containers -- for sample shipment and storage (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests).

15.6.5 Scintillation vials, 20 mL, disposable -- to prepare test concentrations.

15.6.6 Tape, colored -- for labeling tubes.

15.6.7 Markers, waterproof -- for marking containers, etc.

15.6.8 Parafilm -- to cover tubes and vessels containing test materials.

15.6.9 Gloves, disposable; labcoat and protective eyewear – for personal protection from contamination.

15.6.10 Data sheets (one set per test) -- for data recording (see Figures 1, 2, and 3).

15.6.11 Acetic acid, 10%, reagent grade, in seawater -- for preparing killed sperm dilutions.

15.6.12 Formalin, 1%, in 2 mL of seawater -- for preserving eggs (see Subsection 15.10.9 Termination of the Test).

15.6.13 Buffers, pH 4, pH 7, and pH 10 (or as per instructions of instrument manufacturer) -- for standards and calibration check (see USEPA Method 150.1, USEPA, 1979b).

15.6.14 Membranes and filling solutions for dissolved oxygen probe (see USEPA Method 360.1, USEPA, 1979b), or reagents -- for modified Winkler analysis.

15.6.15 Laboratory quality assurance samples and standards -- for the above methods.

15.6.16 Reference toxicant solutions -- see Section 4, Quality Assurance.

15.6.17 Reagent water -- defined as distilled or deionized water that does not contain substances which are toxic to the test organisms.

15.6.18 Effluent, receiving water, and dilution water -- see Section 7, Dilution Water, and Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests.

TEST DATE: _____

SAMPLE: _____

COMPLEX EFFLUENT SAMPLE: _____

COLLECTION DATE: _____

SALINITY/ADJUSTMENT: _____

PH/ADJUSTMENT REQUIRED: _____

PHYSICAL CHARACTERISTICS: _____

STORAGE: _____

COMMENTS: _____

_____

_____

_____

SINGLE COMPOUND: _____

SOLVENT (CONC): _____

TEST CONCENTRATIONS: _____

DILUTION WATER: _____

CONTROL WATER: _____

TEST TEMPERATURE: _____

TEST SALINITY: _____

COMMENTS: _____

_____

_____

_____

Figure 1. Data form (1) for fertilization test using sea urchin, *Arbacia punctulata*.

TEST DATE: _____

SAMPLE: _____

_____

SPERM DILUTIONS:

HEMACYTOMETER COUNT, E: _____ x 10^4 = SPM SOLUTION E = _____

SPERM CONCENTRATIONS: SOLUTION E x 40 = SOLUTION A = _____ SPM
SOLUTION E x 20 = SOLUTION B = _____ SPM
SOLUTION E x 5 = SOLUTION D = _____ SPM

SOLUTION SELECTED FOR TEST (= 5 x 10^7 SPM):

DILUTION: SPM/(5 x 10^7) = _____ DF
[(DF) x 10) - 10 = _____ + SW, mL

FINAL SPERM COUNTS = _____

EGG DILUTIONS:

INITIAL EGG COUNT = _____
ORIGINAL EGG STOCK CONCENTRATION = 10X (INITIAL EGG COUNT) = _____
VOLUME OF SW TO ADD TO DILUTE EGG STOCK TO 2000/mL:
EGG COUNT) - 200 = _____
CONTROL WATER TO ADD EGG STOCK, mL = _____
FINAL EGG COUNT = _____

TEST TIMES:

SPERM COLLECTED: _____

EGGS COLLECTED: _____

SPERM ADDED: _____

EGGS ADDED: _____

FIXATIVE ADDED: _____

SAMPLES READ: _____

Figure 2. Data form (2) for fertilization test using sea urchin, *Arbacia punctulata.*

DATE TESTED: _____

SAMPLE: _____

TOTAL AND UNFERTILIZED EGG COUNT AT END OF TEST:

EFFLUENT	REPLICATE VIAL			
CONC (%)	1 TOTAL-UNFERT	2 TOTAL-UNFERT	3 TOTAL-UNFERT	4 TOTAL-UNFERT

STATISTICAL ANALYSIS:

ANALYSIS OF VARIANCE: _____

CONTROL: _____

DIFFERENT FROM CONTROL (P): _____

_____

COMMENTS: _____

_____

_____

_____

Figure 3. Data form (3) for fertilization test using sea urchin, *Arbacia punctulata*.

15.6.18.1 Saline test and dilution water -- the salinity of the test water must be 30‰. The salinity should vary by no more than ± 2‰ among the replicates. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.6.18.2 The overwhelming majority of industrial and sewage treatment effluents entering marine and estuarine systems contain little or no measurable salts. Exposure of sea urchin eggs and sperm to these effluents will require adjustments in the salinity of the test solutions. It is important to maintain a constant salinity across all treatments. In addition it may be desirable to match the test salinity with that of the receiving water. Two methods are available to adjust salinities – hypersaline brine (HSB) derived from natural seawater or artificial sea salts.

15.6.18.3 Hypersaline brine (HSB): HSB has several advantages that make it desirable for use in toxicity testing. It can be made from any high quality, filtered seawater by evaporation, and can be added to the effluent or to deionized water to increase the salinity. HSB derived from natural seawater contains the necessary trace metals, biogenic colloids, and some of the microbial components necessary for adequate growth, survival, and/or reproduction of marine and estuarine organisms, and may be stored for prolonged periods without any apparent degradation. However, if 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ salinity and 70% at 30‰ salinity.

15.6.18.3.1 The ideal container for making HSB from natural seawater is one that (1) has a high surface to volume ratio, (2) is made of a noncorrosive material, and (3) is easily cleaned (fiberglass containers are ideal). Special care should be used to prevent any toxic materials from coming in contact with the seawater being used to generate the brine. If a heater is immersed directly into the seawater, ensure that the heater materials do not corrode or leach any substances that would contaminate the brine. One successful method used is a thermostatically controlled heat exchanger made from fiberglass. If aeration is utilized, use only oil-free air compressors to prevent contamination.

15.6.18.3.2 Before adding seawater to the brine generator, thoroughly clean the generator, aeration supply tube, heater, and any other materials that will be in direct contact with the brine. A good quality biodegradable detergent should be used, followed by several (at least three) thorough deionized water rinses.

15.6.18.3.3 High quality (and preferably high salinity) seawater should be filtered to at least 10 µm before placing into the brine generator. Water should be collected on an incoming tide to minimize the possibility of contamination.

15.6.18.3.4 The temperature of the seawater is increased slowly to 40°C. The water should be aerated to prevent temperature stratification and to increase water evaporation. The brine should be checked daily (depending on the volume being generated) to ensure that the salinity does not exceed 100‰ and that the temperature does not exceed 40°C. Additional seawater may be added to the brine to obtain the volume of brine required.

15.6.18.3.5 After the required salinity is attained, the HSB should be filtered a second time through a 1 mm filter and poured directly into portable containers, (20 L cubitainers or polycarbonate water cooler jugs are suitable). The containers should be capped and labeled with the date the brine was generated and its salinity. Containers of HSB should be stored in the dark and maintained under room temperature until used.

15.6.18.3.6 If a source of HSB is available, test solutions can be made by following the directions below. Thoroughly mix together the deionized water and brine before mixing in the effluent.

15.6.18.3.7 Divide the salinity of the HSB by the expected test salinity to determine the proportion of deionized water to brine. For example, if the salinity of the brine is 100‰ and the test is to be conducted at 30‰, 100‰ divided by 30‰ = 3.3. The proportion of brine is 1 part in 3.3 (one part brine to 2.3 parts deionized water). To make 1 L of seawater at 30‰ salinity from a HSB of 100‰, 300 mL of brine and 700 mL of deionized water are required.

15.6.18.3.8 Table 1 illustrates the preparation of test solutions at 30‰ if they are made by combining effluent (0‰), deionized water and HSB (100‰), or FORTY FATHOMS® sea salts.

15.6.18.4 Artificial sea salts: FORTY FATHOMS® brand sea salts have been used successfully at the EMSL-Cincinnati, for long-term (6-12 months) maintenance of stock cultures of sexually mature sea urchins and to perform the sea urchin fertilization test. GP2 seawater formulation (Table 2) has also been used successfully at ERL-Narragansett, RI.

15.6.18.4.1 Synthetic sea salts are packaged in plastic bags and mixed with deionized water or equivalent. The instructions on the package of sea salts should be followed carefully, and the salts should be mixed in a separate container -- not in the culture tank. The deionized water used in hydration should be in the temperature range of 21-26°C. Seawater made from artificial sea salts is conditioned (Spotte, 1973; Spotte, et al., 1984; Bower, 1983).

15.6.18.4.2 The GP2 reagent grade chemicals (Table 2) should be mixed with deionized (DI) water or its equivalent in a container other than the culture or testing tanks. The deionized water used for hydration should be between 21-26°C. The artificial seawater must be conditioned (aerated) for 24 h before use as the testing medium. If the solution is to be autoclaved, sodium bicarbonate is added after the solution has cooled. A stock solution of sodium bicarbonate is made up by dissolving 33.6 g $NaHCO_3$ in 500 mL of deionized water. Add 2.5 mL of this stock solution for each liter of the GP2 artificial seawater.

TABLE 1. PREPARATION OF TEST SOLUTIONS AT A SALINITY OF 30‰ USING NATURAL SEAWATER, HYPERSALINE BRINE, OR ARTIFICIAL SEA SALTS [1]

		Solutions To Be Combined	
Effluent Solution	Effluent Concentration (%)	Volume of Effluent Solution (mL)	Volume of Diluent Seawater (30‰) (mL)
1	100[1]	840	—
2	50	420	Solution 1 + 420
3	25	420	Solution 2 + 420
4	12.5	420	Solution 3 + 420
5	6.25	420	Solution 4 + 420
Control	0.0		420
Total			2080

[1] This illustration assumes: (1) the use of 5 mL of test solution in each of four replicates (total of 20 mL) for the control and five concentrations of effluent, (2) an effluent dilution factor of 0.5, (3) the effluent lacks appreciable salinity, and (4) 400 mL of each test concentration is used for chemical analysis. A sufficient initial volume (840 mL) of effluent is prepared by adjusting the salinity to 30‰. In this example, the salinity is adjusted by adding artificial sea salts to the 100% effluent, and preparing a serial dilution using 30‰ seawater (natural seawater, hypersaline brine, or artificial seawater). Stir solutions 1 h to ensure that the salts dissolve. The salinity of the initial 840 mL of 100% effluent is adjusted to 30‰ by adding 25.2 g of dry artificial sea salts (FORTY FATHOMS®). Test concentrations are then made by mixing appropriate volumes of salinity adjusted effluent and 30‰ salinity dilution water to provide 840 mL of solution for each concentration. If hypersaline brine alone (100‰) is used to adjust the salinity of the effluent, the highest concentration of effluent that could be tested would be 70% at 30‰ salinity.

TABLE 2. REAGENT GRADE CHEMICALS USED IN THE PREPARATION OF GP2 ARTIFICIAL SEAWATER FOR THE SEA URCHIN, *ARBACIA PUNCTULATA*, TOXICITY TEST[1,2,3]

Compound	Concentration (g/L)	Amount (g) Required for 20 L
NaCl	21.03	420.6
Na_2SO_4	3.52	70.4
KCl	0.61	12.2
KBr	0.088	1.76
$Na_2B_4O_7 \cdot 10\ H_2O$	0.034	0.68
$MgCl_2 \cdot 6\ H_2O$	9.50	190.0
$CaCl_2 \cdot 2\ H_2O$	1.32	26.4
$SrCl_2 \cdot 6\ H_2O$	0.02	0.400
$NaHCO_3$	0.17	3.40

[1] Modified GP2 from Spotte et al. (1984).
[2] The constituent salts and concentrations were taken from USEPA (l990b). The salinity is 30.89 g/L.
[3] GP2 can be diluted with deionized (DI) water to the desired test salinity.

15.6.19 TEST ORGANISMS, SEA URCHINS, *ARBACIA PUNCTULATA*

15.6.19.1 Adult sea urchins, *Arbacia punctulata*, can be obtained from commercial suppliers. After acquisition, the animals are sexed by briefly stimulating them with current from a 12 V transformer. Electrical stimulation causes the immediate release of masses of gametes that are readily identifiable by color -- the eggs are red, and the sperm are white.

15.6.19.2 The sexes are separated and maintained in 20-L, aerated fiberglass tanks, each holding about 20 adults. The tanks are supplied continuously (approximately 5 L/min) with filtered natural seawater, or salt water prepared from commercial sea salts is recirculated. The animals are checked daily and any obviously unhealthy animals are discarded.

15.6.19.3 The culture unit should be maintained at 15 ± 3°C, with a water temperature control device.

15.6.19.4 The food consists of kelp, *Laminaria* sp., gathered from known uncontaminated zones or obtained from commercial supply houses whose kelp comes from known uncontaminated areas, or romaine lettuce. Fresh food is introduced into the tanks at approximately one week intervals. Decaying food is removed as necessary. Ample supplies of food should always be available to the sea urchins.

15.6.19.5 Natural or artificial seawater with a salinity of 30‰ is used to maintain the adult animals, for all washing and dilution steps, and as the control water in the tests (see Subsection 15.6.18).

15.6.19.6 Adult male and female animals used in field studies are transported in separate or partitioned insulated boxes or coolers packed with wet kelp or paper toweling. Upon arrival at the field site, aquaria (or a single partitioned

aquarium) are filled with control water, loosely covered with a styrofoam sheet and allowed to equilibrate to 15°C before animals are added. Healthy animals will attach to the kelp or aquarium within hours.

15.6.19.7 To successfully maintain about 25 adult animals for 7 days at a field site, a screen-partitioned, 40-L glass aquarium using aerated, recirculating, clean saline water (30‰) and a gravel bed filtration system, is housed within a water bath, such as FORTY FATHOMS® or equivalent (15°C). The inner aquarium is used to avoid contact of animals and water bath with cooling coils.

15.7 EFFLUENT AND RECEIVING WATER COLLECTION, PRESERVATION, AND STORAGE

15.7.1 See Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sampling Preparation for Toxicity Tests.

15.8 CALIBRATION AND STANDARDIZATION

15.8.1 See Section 4, Quality Assurance.

15.9 QUALITY CONTROL

15.9.1 See Section 4, Quality Assurance.

15.10 TEST PROCEDURES

15.10.1 TEST SOLUTIONS

15.10.1.1 Receiving Waters

15.10.1.1.1 The sampling point is determined by the objectives of the test. At estuarine and marine sites, samples are usually collected at mid-depth. Receiving water toxicity is determined with samples used directly as collected or with samples passed through a 60 µm NITEX® filter and compared without dilution against a control. Using four replicate chambers per test, each containing 5 mL, and 400 mL for chemical analysis, would require approximately 420 mL or more of sample per test.

15.10.1.2 Effluents

15.10.1.2.1 The selection of the effluent test concentrations should be based on the objectives of the study. A dilution factor of 0.5 is commonly used. A dilution factor of 0.5 provides precision of ± 100%, and testing of concentrations between 6.25% and 100% effluent using only five effluent concentrations (6.25%, 12.5%, 25%, 50%, and 100%). Test precision shows little improvement as dilution factors are increased beyond 0.5 and declines rapidly if smaller dilution factors are used. **Therefore, USEPA recommends the use of the ≥ 0.5 dilution factor.** If 100‰ HSB is used as a diluent, the maximum concentration of effluent that can be tested will be 80% at 20‰ and 70% at 30‰ salinity.

15.10.1.2.2 If the effluent is known or suspected to be highly toxic, a lower range of effluent concentrations should be used (such as 25%, 12.5%, 6.25%, 3.12%, and 1.56%).

15.10.1.2.3 Just prior to test initiation (approximately 1 h), a sufficient quantity of the sample to make the test solutions should be adjusted to the test temperature (20 ± 1°C) and maintained at that temperature during the addition of dilution water.

15.10.1.2.4 The test should begin as soon as possible, preferably within 24 h of sample collection. The maximum holding time following retrieval of the sample from the sampling device should not exceed 36 h for off-site toxicity tests unless permission is granted by the permitting authority. In no case should the sample be used for the first time in a test more than 72 h after sample collection (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Test).

15.10.1.2.5 Effluent dilutions should be prepared for all replicates in each treatment in one beaker to minimize variability among the replicates. The test chambers are labeled with the test concentration and replicate number. Dispense into the appropriate effluent dilution chamber.

15.10.1.3 Dilution Water

15.10.1.3.1 Dilution water may be uncontaminated natural seawater (receiving water), HSB prepared from natural seawater, or artificial seawater FORTY FATHOMS® or GP2 sea salts (see Table 2 and Section 7, Dilution Water). Prepare 3 L of control water at 30‰ using HSB or artificial sea salts (see Table 1). This water is used in all washing and diluting steps and as control water in the test. Natural seawater and local waters may be used as additional controls.

15.10.2 COLLECTION OF GAMETES FOR THE TEST

15.10.2.1 Select four females and place in shallow bowls, barely covering the shell with seawater. Stimulate the release of eggs by touching the shell with steel electrodes connected to a 10-12 volt transformer (about 30 seconds each time). Collect the eggs from each female using a 10 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle (tip cut off). Remove the needle from the syringe before adding the eggs to a conical centrifuge tube. Pool the eggs. The egg stock may be held at room temperature for several hours before use. Note: Eggs should be collected first to eliminate possibility of pre-fertilization.

15.10.2.2 Select four males and place in shallow bowls, barely covering the animals with seawater. Stimulate the release of sperm as described above. Collect the sperm (about 0.25 mL) from each male, using a 1-3 mL disposable syringe fitted with an 18-gauge, blunt-tipped needle. Pool the sperm. Maintain the pooled sperm sample on ice. The sperm must be used in a toxicity test within 1 h of collection.

15.10.3 PREPARATION OF SPERM DILUTION FOR USE IN THE TEST

15.10.3.1 Using control water, dilute the pooled sperm sample to a concentration of about 5 X $l0^7$ sperm/mL (SPM). Estimate the sperm concentration as described below:

1. Make a sperm dilutions of 1:50, 1:100, 1:200, and 1:400, using 30‰ seawater, as follows:

    a. Add 400 µL of collected sperm to 20 mL of seawater in Vial A. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    b. Add 10 mL of sperm suspension from Vial A to 10 mL of seawater in Vial B. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    c. Add 10 mL of sperm suspension from Vial B to 10 mL of seawater in Vial C. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    d. Add 10 mL of sperm suspension from Vial C to 10 mL of seawater in Vial D. Mix by gentle pipetting using a 5-mL pipettor, or by inversion;
    e. Discard 10 mL from Vial D. (The volume of all suspensions is 10 mL).

2. Make a 1:2000 killed sperm suspension and determine the SPM.

    a. Add 10 mL 10% acetic acid in seawater to Vial C. Cap Vial C and mix by inversion.
    b. Add 1 mL of killed sperm from Vial C to 4 mL of seawater in Vial E. Mix by gentle pipetting with a 4-mL pipettor.
    c. Add sperm from Vial E to both sides of the Neubauer hemacytometer. Let the sperm settle 15 min.
    d. Count the number of sperm in the central 400 squares on both sides of the hemacytometer using a compound microscope (100X). Average the counts from the two sides.
    e. SPM in Vial E = 10^4 x average count.

3. Calculate the SPM in all other suspensions using the SPM in Vial E above:

   SPM in Vial A = 40 x SPM in Vial E
   SPM in Vial B = 20 x SPM in Vial E
   SPM in Vial D = 5 x SPM in Vial E
   SPM in original sperm sample = 2000 x SPM in Vial E

4. Dilute the sperm suspension with a SPM greater than 5×10^7 SPM to 5×10^7 SPM.

   Actual SPM/(5×10^7) = dilution factor (DF)

   [(DF) x 10] - 10 = mL of seawater to add to vial.

5. Confirm the sperm count by sampling from the test stock. Add 0.1 mL of test stock to 9.9 mL of 10% acetic acid in seawater, and count with the hemacytometer. The count should average 50 ± 5.

15.10.4 PREPARATION OF EGG SUSPENSION FOR USE IN THE TEST Note: The egg suspension may be prepared during the 1-h sperm exposure.

15.10.4.1 Wash the pooled eggs three times using control water with gentle centrifugation (500xg for 3 minutes using a tabletop centrifuge). If the wash water becomes red, the eggs have lysed and must be discarded.

15.10.4.2 Dilute the egg stock, using control water, to about 2000 eggs/mL.

1. Transfer the eggs to a glass beaker containing 200 mL of control water ("egg stock").

2. Mix the egg stock using an air-bubbling device. Using a wide-mouth pipet tip, transfer 1 mL of eggs from the egg stock to a vial containing 9 mL of control water. (This vial contains an egg suspension diluted 1:10 from egg stock).

3. Mix the contents of the vial by inversion. Using a wide-mouth pipet tip, transfer 1 mL of eggs from the vial to a Sedgwick-Rafter counting chamber. Count all eggs in the chamber using a dissecting microscope at 24X "egg count".

4. Calculate the concentration of eggs in the stock. Eggs/mL = 10X (egg count). Dilute the egg stock to 2000 eggs/mL by the formula below.

   a. If the egg count is equal to or greater than 200:
      (egg count) - 200 = volume (mL) of control water to add to egg stock.
   b. If the egg count is less than 200, allow the eggs to settle and remove enough control water to concentrate the eggs to greater than 200, repeat the count, and dilute the egg stock as in a. above. NOTE: It requires 24 mL of a egg stock solution for each test with a control and five exposure concentrations.
   c. Transfer 1 mL of the diluted egg stock to a vial containing 9 mL of control water. Mix well, then transfer 1 mL from the vial to a Sedgwick-Rafter counting chamber. Count all eggs using a dissecting microscope. Confirm that the final egg count = 2000/mL (± 200).

15.10.5 LIGHT, PHOTOPERIOD, SALINITY AND TEMPERATURE

15.10.5.1 The light quality and intensity should be at ambient laboratory levels 10-20 $\mu E/m^2/s$ (50-100 ft-c) with a photoperiod of 16 h light and 8 h darkness. The water temperature in the test chambers should be maintained at 20 ± 1°C. The test salinity should be in the range of 28 to 32‰. The salinity should vary by no more that ± 2‰ among the chambers on a given day. If effluent and receiving water tests are conducted concurrently, the salinities of these tests should be similar.

15.10.6 DISSOLVED OXYGEN (DO) CONCENTRATION

15.10.6.1 Aeration may affect the toxicity of effluent and should be used only as a last resort to maintain a satisfactory DO. The DO concentrations should be measured on new solutions at the start of the test (Day 0). The DO should not fall below 4.0 mg/L (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests). If it is necessary to aerate, all treatments and the control should be aerated. The aeration rate should not exceed 100 bubbles/minute, using a pipet with a 1-2 mm orifice, such as a 1 mL KIMAX® serological pipet, or equivalent.

15.10.7 OBSERVATIONS DURING THE TEST

15.10.7.1 Routine Chemical and Physical Observations

15.10.7.1.1 DO is measured at the beginning of the exposure period in one test chamber at each test concentration and in the control.

15.10.7.1.2 Temperature, pH, and salinity are measured at the beginning of the exposure period in one test chamber at each concentration and in the control. Temperature should also be monitored continuously observed and recorded daily for at least two locations in the environmental control system or the samples. Temperature should be measured in a sufficient number of test chambers at least at the end of the test to determine temperature variation in environmental chamber.

15.10.7.1.3 The pH is measured in the effluent sample each day before new test solutions are made.

15.10.7.1.4 Record all the measurements on the data sheet.

15.10.7.2 Routine Biological Observations

15.10.7.2.1 Fertilization will be determined by the presence of a fertilization membrane surrounding the egg.

15.10.8 START OF THE TEST

15.10.8.1 Effluent/receiving water samples are adjusted to salinity of 30‰. Four replicates are prepared for each test concentration, using 5 mL of solution in disposable liquid scintillation vials. A 50% (0.5) concentration series can be prepared by serially diluting test concentrations with control water. Sufficient test solution is prepared at each effluent concentration to provide additional volume for chemical analyses, at the high, medium, and low test concentrations.

15.10.8.2 All test samples are equilibrated at 20°C ± 1°C before addition of sperm.

15.10.8.3 Within 1 h of collection add 100 µL of appropriately diluted sperm to each test vial. Record the time of sperm addition.

15.10.8.4 Incubate all test vials at 20 ± 1°C for 1 h.

15.10.8.5 Mix the diluted egg suspension (2000 eggs/mL), using gentle bubbling. Add 1 mL of diluted egg suspension to each test vial using a wide mouth pipet tip. Incubate 20 min at 20 ± 1°C.

15.10.9 TERMINATION OF THE TEST

15.10.9.1 Terminate the test and preserve the samples by adding 2 mL of 1% formalin in seawater to each vial.

15.10.9.2 Vials should be evaluated within 48 hours.

15.10.9.3 To determine fertilization, transfer about 1 mL eggs from the bottom of a test vial to a Sedgwick-Rafter counting chamber. Observe the eggs using a compound microscope (100X). Count between 100 and 200 eggs/sample. Record the number counted and the number unfertilized. Fertilization is indicated by the presence of a fertilization membrane surrounding the egg. NOTE: adjustment of the microscope to obtain proper contrast may be required to observe the fertilization membrane. Because samples are fixed in formalin, a ventilation hood is set up surrounding the microscope to protect the analyst from prolonged exposure to formaldehyde fumes.

15.11 SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA

15.11.1 A summary of test conditions and test acceptability criteria is listed in Table 3.

15.12 ACCEPTABILITY OF TEST RESULTS

15.12.1 The sperm:egg ratio routinely employed must result in fertilization of 70%-90% of the eggs in the control chambers.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST WITH EFFLUENT AND RECEIVING WATERS (TEST METHOD 1008.0)[1]

1	Test type:	Static (required)
2.	Salinity:	30‰ (± 2‰ of the selected test salinity) (recommended)
3.	Temperature:	20 ± 1°C (recommended) Test temperatures must not deviate (i.e., maximum minus minimum temperature) by more than 3°C during the test (required)
4.	Light quality:	Ambient laboratory light during test preparation (recommended)
5.	Light intensity:	10-20 µE/m^2/s, or 50-100 ft-c (Ambient laboratory levels) (recommended)
6.	Test chamber size:	Disposable (glass) liquid scintillation vials (20 mL capacity), presoaked in control water (recommended)
7.	Test solution volume:	5 mL (recommended)
8.	No. of sea urchins:	Pooled sperm from four males and pooled eggs from four females are used per test (recommended)
9.	No. egg and sperm cells per chamber:	About 2,000 eggs and 5,000,000 sperm cells per vial (recommended)
10.	No. replicate chambers per concentration:	4 (required minimum)
11.	Dilution water:	Uncontaminated source of natural seawater; deionized water mixed with hypersaline brine or artificial sea salts (HW MARINEMIX®, FORTY FATHOMS®, GP2, or equivalent) (available options)
12.	Test concentrations:	Effluents: 5 and a control (required minimum) Receiving waters: 100% receiving water (or minimum of 5) and a control (recommended)

[1] For the purposes of reviewing WET test data submitted under NPDES permits, each test condition listed above is identified as required or recommended (see Subsection 10.2 for more information on test review). Additional requirements may be provided in individual permits, such as specifying a given test condition where several options are given in the method.

TABLE 3. SUMMARY OF TEST CONDITIONS AND TEST ACCEPTABILITY CRITERIA FOR SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST WITH EFFLUENT AND RECEIVING WATERS (TEST METHOD 1008.0) (CONTINUED)

13. Dilution factor:	Effluents: ≥0.5 (recommended) Receiving waters: None or ≥0.5 (recommended)
14. Test duration:	1 h and 20 min (required)
15. Endpoint:	Fertilization of sea urchin eggs (required)
16. Test acceptability criteria:	70% - 90% egg fertilization in controls (required)
17. Sampling requirements:	For on-site tests, one sample collected at test initiation, and used within 24 h of the time it is removed from the sampling device. For off-site tests, holding time must not exceed 36 h before first use (see Section 8, Effluent and Receiving Water Sampling, Sample Handling, and Sample Preparation for Toxicity Tests, Subsection 8.5.4) (required)
18. Sample volume required:	1 L per test (recommended)

15.13 DATA ANALYSIS

15.13.1 GENERAL

15.13.1.1 Tabulate and summarize the data. Calculate the proportion of fertilized eggs for each replicate. A sample set of test data is listed in Table 4.

15.13.1.2 The statistical tests described here must be used with a knowledge of the assumptions upon which the tests are contingent. The assistance of a statistician is recommended for analysts who are not proficient in statistics.

TABLE 4. DATA FROM SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST

Copper Concentration (µg/L)	Replicate	No. of Eggs Counted	No. of Eggs Fertilized	Proportion Fertilized
Control	A	100	85	0.85
	B	100	78	0.78
	C	100	87	0.87
2.5	A	100	81	0.81
	B	100	65	0.65
	C	100	71	0.71
5.0	A	100	63	0.63
	B	100	74	0.74
	C	100	78	0.78
10.0	A	100	63	0.63
	B	100	66	0.66
	C	100	51	0.51
20.0	A	100	41	0.41
	B	100	41	0.41
	C	100	37	0.37
40.0	A	100	12	0.12
	B	100	30	0.30
	C	100	26	0.26

[1] Tests performed by Dennis M. McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.

15.13.1.3 The endpoints of toxicity tests using the sea urchin are based on the reduction in proportion of eggs fertilized. The IC25 and the IC50 are calculated using the Linear Interpolation Method (see Section 9, Chronic Toxicity Test Endpoints and Data Analysis). LOEC and NOEC values for fecundity are obtained using a hypothesis testing approach such as Dunnett's Procedure (Dunnett, 1955) or Steel's Many-one Rank Test (Steel, 1959; Miller, 1981) (see Section 9). Separate analyses are performed for the estimation of the LOEC and NOEC endpoints and for the estimation of IC25 and IC50. See the Appendices for examples of the manual computations, and examples of data input and program output.

15.13.2 EXAMPLE OF ANALYSIS OF SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION DATA

15.13.2.1 Formal statistical analysis of the fertilization data is outlined in Figure 4. The response used in the analysis is the proportion of fertilized eggs in each test or control chamber. Separate analyses are performed for the estimation of the NOEC and LOEC endpoints and for the estimation of the IC25 and IC50 endpoints. Concentrations at which there are no eggs fertilized in any of the test chambers are excluded from statistical analysis of the NOEC and LOEC, but included in the estimation of the IC25 and IC50.

15.13.2.2 For the case of equal numbers of replicates across all concentrations and the control, the evaluation of the NOEC and LOEC endpoints is made via a parametric test, Dunnett's Procedure, or a nonparametric test, Steel's Many-one Rank Test, on the arc sine square root transformed data. Underlying assumptions of Dunnett's Procedure, normality and homogeneity of variance, are formally tested. The test for normality is the Shapiro-Wilk's Test, and

Bartlett's Test is used to test for homogeneity of variance. If either of these tests fails, the nonparametric test, Steel's Many-one Rank Test, is used to determine the NOEC and LOEC endpoints. If the assumptions of Dunnett's Procedure are met, the endpoints are estimated by the parametric procedure.

15.13.2.3 If unequal numbers of replicates occur among the concentration levels tested, there are parametric and nonparametric alternative analyses. The parametric analysis is a t test with the Bonferroni adjustment (see Appendix D). The Wilcoxon Rank Sum Test with the Bonferroni adjustment is the nonparametric alternative.

15.13.2.4 Example of Analysis of Fecundity Data

15.13.2.4.1 This example uses toxicity data from a sea urchin, *Arbacia punctulata*, fertilization test performed with copper. The response of interest is the proportion of fertilized eggs, thus each replicate must first be transformed by the arc sine square root transformation procedure described in Appendix B. The raw and transformed data, means and variances of the transformed observations at each copper concentration and control are listed in Table 5. The data are plotted in Figure 5.

15.13.2.5 Test for Normality

15.13.2.5.1 The first step of the test for normality is to center the observations by subtracting the mean of all observations within a concentration from each observation in that concentration. The centered observations are summarized in Table 6.

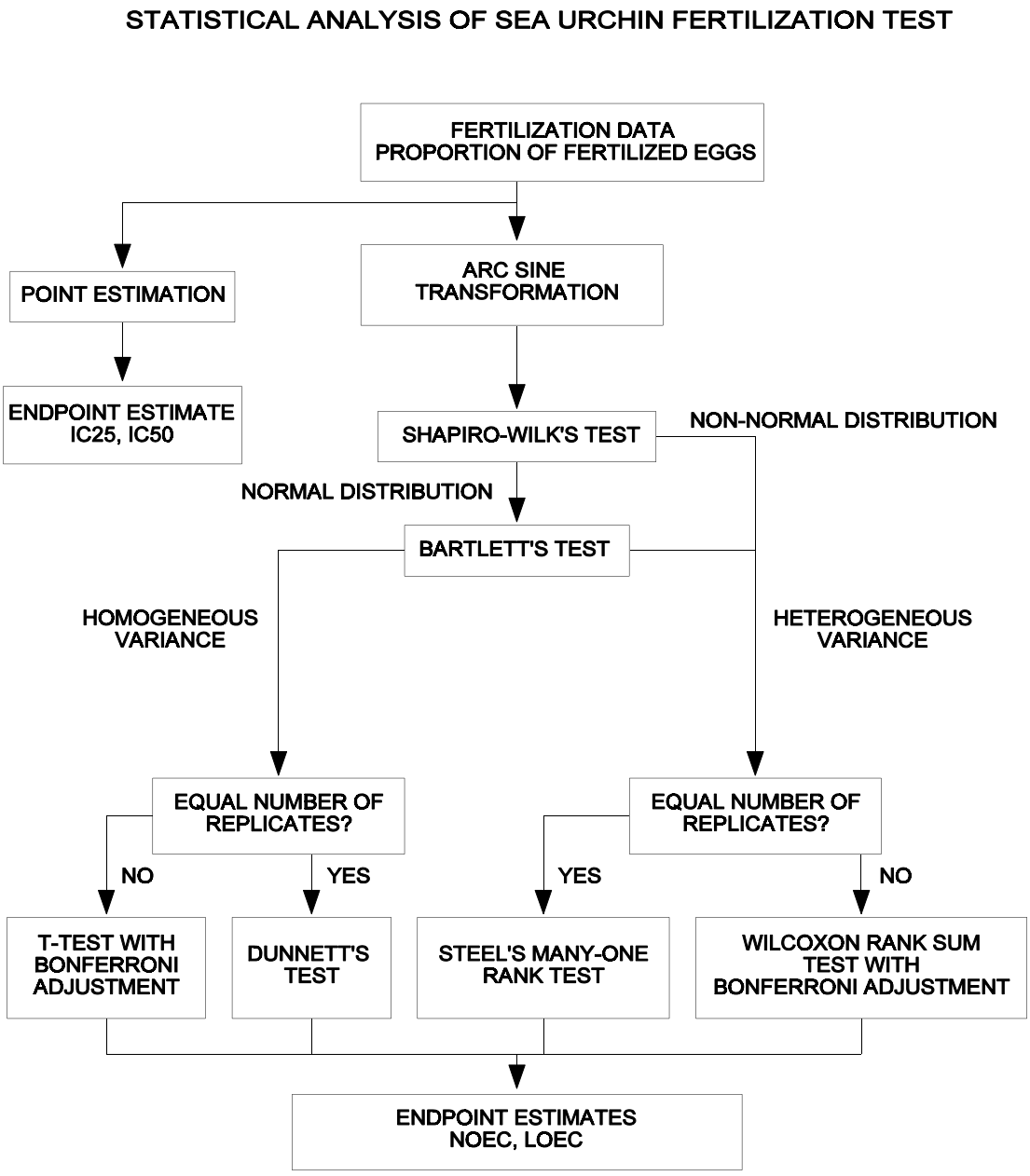

Figure 4. Flowchart for statistical analysis of sea urchin, *Arbacia punctulata*, by point estimation.

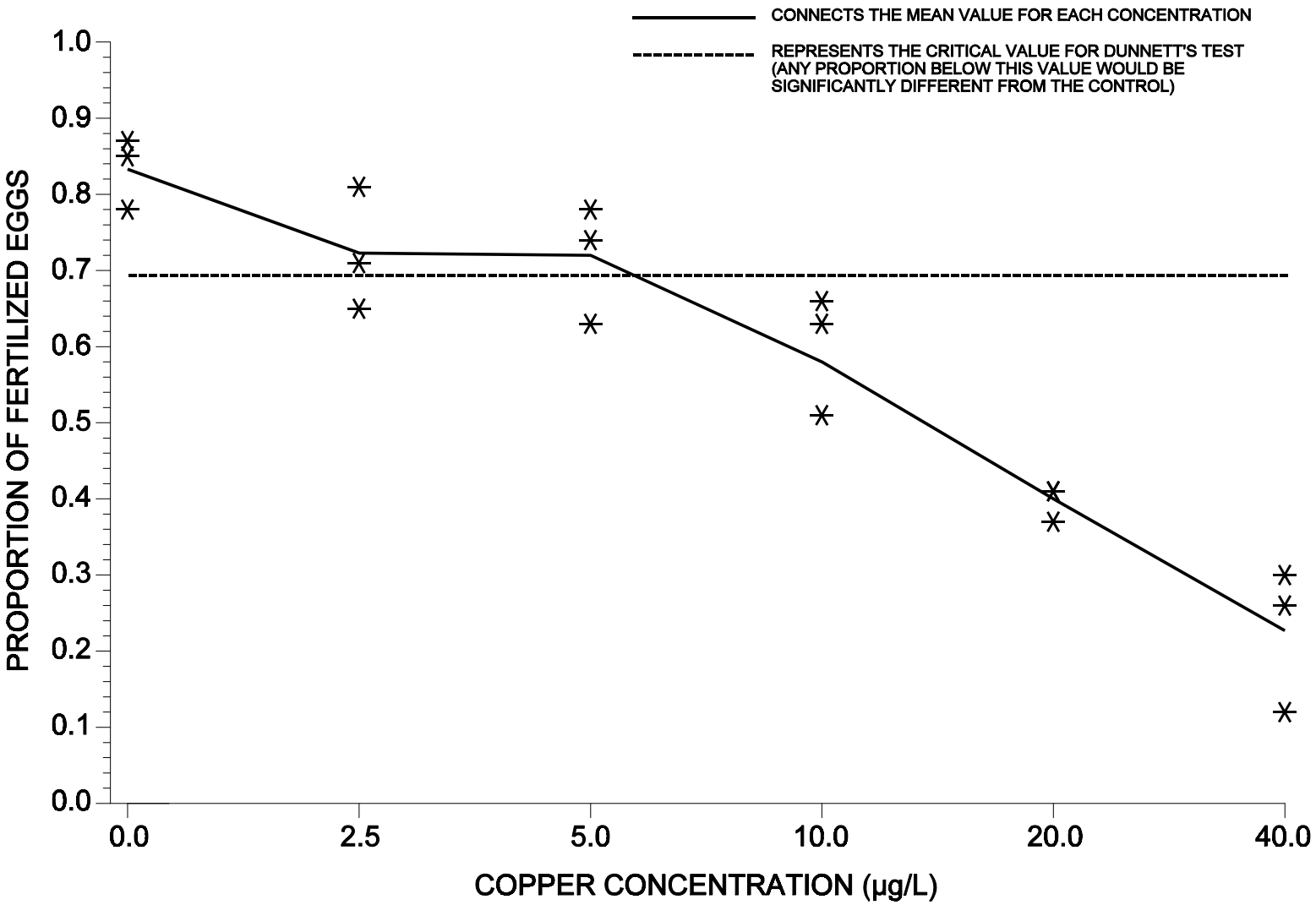

Figure 5. Plot of mean percent of fertilized sea urchin, *Arbacia punctulata*, eggs.

TABLE 5. SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION DATA

		Copper Concentration (µg/L)					
	Replicate	Control	2.5	5.0	10.0	20.0	40.0
	A	0.85	0.81	0.63	0.63	0.41	0.12
RAW	B	0.78	0.65	0.74	0.66	0.41	0.30
	C	0.87	0.71	0.78	0.51	0.37	0.26
ARC SINE	A	1.173	1.120	0.917	0.917	0.695	0.354
TRANSFORMED	B	1.083	0.938	1.036	0.948	0.695	0.580
	C	1.202	1.002	1.083	0.795	0.654	0.535
Mean ($\bar{Y}_i$)		1.153	1.020	1.012	0.887	0.681	0.490
S_i^2		0.004	0.009	0.007	0.007	0.001	0.014
i		1	2	3	4	5	6

TABLE 6. CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

		Copper Concentration (µg/L)				
Replicate	Control	2.5	5.0	10.0	20.0	40.0
A	0.020	0.100	-0.095	0.030	0.014	-0.136
B	-0.070	-0.082	0.024	0.061	0.014	0.090
C	0.049	-0.018	0.071	-0.092	-0.027	0.045

15.13.2.5.2 Calculate the denominator, D, of the statistic:

$$D = \sum_{i=1}^{n} (X_i - \bar{X})^2$$

Where: X_i = the ith centered observation

$\bar{X}$ = the overall mean of the centered observations

n = the total number of centered observations

15.13.2.5.3 For this set of data, n = 18

$$\bar{X} = \frac{1}{18}(0) = 0$$

$$D = 0.0822$$

15.13.2.5.4 Order the centered observations from smallest to largest

$$X^{(1)} \le X^{(2)} \le \ldots \le X^{(n)}$$

where $X^{(i)}$ denotes the ith ordered observation. The ordered observations for this example are listed in Table 7.

TABLE 7. ORDERED CENTERED OBSERVATIONS FOR SHAPIRO-WILK'S EXAMPLE

i	$X^{(i)}$	i	$X^{(i)}$
1	-0.136	10	0.020
2	-0.095	11	0.024
3	-0.092	12	0.030
4	-0.082	13	0.045
5	-0.070	14	0.049
6	-0.027	15	0.061
7	-0.018	16	0.071
8	0.014	17	0.090
9	0.014	18	0.100

15.13.2.5.5 From Table 4, Appendix B, for the number of observations, n, obtain the coefficients $a_1, a_2, \ldots a_k$ where k is n/2 if n is even and (n-1)/2 if n is odd. For the data in this example, n = 18 and k = 9. The a_i values are listed in Table 8.

15.13.2.5.6 Compute the test statistic, W, as follows:

$$W = \frac{1}{D}\left[\sum_{i=1}^{k} a_i (X^{(n-i+1)} - X^{(i)})\right]^2$$

The differences, $X^{(n-i+1)} - X^{(i)}$, are listed in Table 8. For the data in this example:

$$W = \frac{1}{0.0822}(0.2782)^2 = 0.942$$

15.13.2.5.7 The decision rule for this test is to compare W as calculated in Subsection 15.13.2.5.6 to a critical value found in Table 6, Appendix B. If the computed W is less than the critical value, conclude that the data are not normally distributed. For the data in this example, the critical value at a significance level of 0.01 and n = 18 observations is 0.858. Since W = 0.942 is greater than the critical value, conclude that the data are normally distributed.

TABLE 8. COEFFICIENTS AND DIFFERENCES FOR SHAPIRO-WILK'S EXAMPLE

i	a_i	$X^{(n-i+1)} - X^{(i)}$	
1	0.4886	0.236	$X^{(18)} - X^{(1)}$
2	0.3253	0.185	$X^{(17)} - X^{(2)}$
3	0.2553	0.163	$X^{(16)} - X^{(3)}$
4	0.2027	0.143	$X^{(15)} - X^{(4)}$
5	0.1587	0.119	$X^{(14)} - X^{(5)}$
6	0.1197	0.072	$X^{(13)} - X^{(6)}$
7	0.0837	0.048	$X^{(12)} - X^{(7)}$
8	0.0496	0.010	$X^{(11)} - X^{(8)}$
9	0.0163	0.006	$X^{(10)} - X^{(9)}$

15.13.2.6 Test for Homogeneity of Variance

15.13.2.6.1 The test used to examine whether the variation in the proportion of fertilized eggs is the same across all copper concentrations including the control, is Bartlett's Test (Snedecor and Cochran, 1980). The test statistic is as follows:

$$B = \frac{[(\sum_{i=1}^{p} V_i)\ \ln \overline{S}^2 - \sum_{i=1}^{p} V_i\ \ln\ S_i^2]}{C}$$

Where: V_i = degrees of freedom for each copper concentration and control, $V_i = (n_i - 1)$

p = number of levels of copper concentration including the control

n_i = the number of replicates for concentration i.

ln = $\log_e$

i = 1,2, ..., p where p is the number of concentrations including the control

$$\overline{S}^2 = \frac{(\sum_{i=1}^{p} V_i S_i^2)}{\sum_{i=1}^{p} V_i}$$

$$C = 1 + [3(p-1)]^{-1}[\sum_{i=1}^{p} 1/V_i - (\sum_{i=1}^{p} V_i)^{-1}]$$

15.13.2.6.2 For the data in this example (see Table 5), all copper concentrations including the control have the same number of replicates ($n_i = 3$ for all i). Thus, $V_i = 2$ for all i.

15.13.2.6.3 Bartlett's statistic is, therefore:

$$B = [(12)\ln(0.0007) - 2\sum_{i=1}^{p} \ln(S_i^2)]/1.194$$

$$= [12(-4.962) - 2(-31.332)]/1.194$$

$$= 3.122/1.194$$

$$= 2.615$$

15.13.2.6.4 B is approximately distributed as chi-square with p-1 degrees of freedom, when the variances are in fact the same. Therefore, the appropriate critical value for this test, at a significance level of 0.01 with 5 degrees of freedom, is 15.09. Since B = 2.615 is less than the critical value of 15.09, conclude that the variances are not different.

15.13.2.7 Dunnett's Procedure

15.13.2.7.1 To obtain an estimate of the pooled variance for the Dunnett's Procedure, construct an ANOVA table as described in Table 9.

TABLE 9. ANOVA TABLE

Source	df	Sum of Squares (SS)	Mean Square (MS) (SS/df)
Between	p - 1	SSB	S_B^2 = SSB/(p-1)
Within	N - p	SSW	S_W^2 = SSW/(N-p)
Total	N - 1	SST	

Where: p = number of concentration levels including the control

N = total number of observations $n_1 + n_2 ... + n_p$

n_i = number of observations in concentration i

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N \qquad \text{Between Sum of Squares}$$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N \qquad \text{Total Sum of Squares}$$

$SSW = SST - SSB$ Within Sum of Squares

G = the grand total of all sample observations, $G = \sum_{i=1}^{p} T_i$

T_i = the total of the replicate measurements for concentration i

Y_{ij} = the jth observation for concentration i (represents the proportion of fertilized eggs for upper concentration i in test chamber j)

15.13.2.7.2 For the data in this example:

$n_1 = n_2 = n_3 = n_4 = n_5 = n_6 = 3$

$N = 18$

$T_1 = Y_{11} + Y_{12} + Y_{13} = 3.458$
$T_2 = Y_{21} + Y_{22} + Y_{23} = 3.060$
$T_3 = Y_{31} + Y_{32} + Y_{33} = 3.036$
$T_4 = Y_{41} + Y_{42} + Y_{43} = 2.660$
$T_5 = Y_{51} + Y_{52} + Y_{53} = 2.044$
$T_6 = Y_{61} + Y_{62} + Y_{63} = 1.469$

$G = T_1 + T_2 + T_3 + T_4 + T_5 + T_6 = 15.727$

$$SSB = \sum_{i=1}^{p} T_i^2/n_i - G^2/N$$

$= (43.950)/3 - (15.727)^2/18 = 0.909$

$$SST = \sum_{i=1}^{p} \sum_{j=1}^{n_i} Y_{ij}^2 - G^2/N$$

$= 14.732 - (15.727)^2/18 = 0.991$

$SSW = SST - SSB$

$= 0.991 - 0.909 = 0.082$

$S_B^2 = SSB/(p-1) = 0.909/(6-1) = 0.182$

$S_W^2 = SSW/(N-p) = 0.082/(18-6) = 0.007$

15.13.2.7.3 Summarize these calculations in the ANOVA table (Table 10).

TABLE 10. ANOVA TABLE FOR DUNNETT'S PROCEDURE EXAMPLE

Source	df	Sum of Squares (SS)	Mean Square(MS) (SS/df)
Between	5	0.909	0.182
Within	12	0.082	0.007
Total	17	0.991	

15.13.2.7.4 To perform the individual comparisons, calculate the t statistic for each concentration, and control combination as follows:

$$t_i = \frac{(\bar{Y}_1 - \bar{Y}_i)}{S_w\sqrt{(1/n_1) + (1/n_i)}}$$

Where: $\bar{Y}_i$ = mean proportion fertilized eggs for copper concentration i

$\bar{Y}_1$ = mean proportion fertilized eggs for the control

S_W = square root of the within mean square

n_1 = number of replicates for the control

n_i = number of replicates for concentration i

Since we are looking for a decreased response from the control in the proportion of fertilized eggs, the concentration mean is subtracted from the control mean.

15.13.2.7.5 Table 11 includes the calculated t values for each concentration and control combination. In this example, comparing the 2.5 µg/L concentration with the control the calculation is as follows:

$$t_2 = \frac{(1.153 - 1.020)}{[0.084\sqrt{(1/3) + (1/3)}]} = 1.939$$

TABLE 11. CALCULATED T VALUES

Copper Concentration (µg/L)	i	t_i
2.5	2	1.939
5.0	3	2.056
10.0	4	3.878
20.0	5	6.882
40.0	6	9.667

15.13.2.7.6 Since the purpose of this test is to detect a significant decrease in the proportion of fertilized eggs, a one-sided test is appropriate. The critical value for this one-sided test is found in Table 5, Appendix D. For an overall alpha level of 0.05, 12 degrees of freedom for error and five concentrations (excluding the control) the critical value is 2.50. The mean proportion of fertilized eggs for concentration i is considered significantly less than the mean proportion of fertilized eggs for the control if t_i is greater than the critical value. Therefore, the 10.0 µg/L, 20.0 µg/L and 40.0 µg/L concentrations have a significantly lower mean proportion of fertilized eggs than the control. Hence the NOEC is 5.0 µg/L and the LOEC is 10.0 µg/L.

15.13.2.7.7 To quantify the sensitivity of the test, the minimum significant difference (MSD) that can be statistically detected may be calculated:

$$MSD = d\ S_w\sqrt{(1/n_1) + (1/n)}$$

Where: d = the critical value for Dunnett's Procedure

S_W = the square root of the within mean square

n = the common number of replicates at each concentration (this assumes equal replication at each concentration)

n_1 = the number of replicates in the control.

15.13.2.7.8 In this example,

$$MSD = 2.50\,(0.084)\sqrt{(1/3) + (1/3)}$$

$$= 2.50\ (0.084)(0.816)$$

$$= 0.171$$

15.13.2.7.9 The MSD (0.171) is in transformed units. To determine the MSD in terms of proportion of fertilized eggs, carry out the following conversion.

1. Subtract the MSD from the transformed control mean.

$$1.153 - 0.171 = 0.982$$

2. Obtain the untransformed values for the control mean and the difference calculated in step 1 of 15.13.2.7.9.1

$$[\ \text{Sine}\ (1.153)\ ]^2 = 0.835$$

$$[\text{ Sine } (0.982)\text{ }]^2 = 0.692$$

3. The untransformed MSD (MSD_u) is determined by subtracting the untransformed values from step 2 in 15.13.2.7.9.

$$MSD_u = 0.835 - 0.692 = 0.143$$

15.13.2.7.10 Therefore, for this set of data, the minimum difference in mean proportion of fertilized eggs between the control and any copper concentration that can be detected as statistically significant is 0.143.

15.13.2.7.11 This represents a 17% decrease in the proportion of fertilized eggs from the control.

15.13.2.8 Calculation of the ICp

15.13.2.8.1 The fertilization data in Table 4 are utilized in this example. Table 12 contains the mean proportion of fertilized eggs for each toxicant concentration. As can be seen, the observed means are monotonically non-increasing with respect to concentration. Therefore, it is not necessary to smooth the means prior to calculating the ICp; (see Figure 5 for a plot of the response curve).

15.13.2.8.2 An IC25 and IC50 can be estimated using the Linear Interpolation Method. A 25% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.625, where $M_1(1-p/100) = 0.833(1-25/100)$. A 50% reduction in mean proportion of fertilized eggs, compared to the controls, would result in a mean proportion of 0.417. Examining the means and their associated concentrations (Table 12), the response, 0.625, is bracketed by $C_3 = 5.0$ µg/L copper and $C_4 = 10.0$ µg/L copper. The response, 0.417, is bracketed by $C_4 = 10.0$ µg/L copper and $C_5 = 20.0$ µg/L copper.

TABLE 12. SEA URCHIN, *ARBACIA PUNCTULATA*, MEAN PROPORTION OF FERTILIZED EGGS

Copper Conc. (µg/L)	i	Response Means Y_i (proportion)	Smoothed Mean M_i (proportion)
Control	1	0.833	0.833
2.5	2	0.723	0.723
5.0	3	0.717	0.717
10.0	4	0.600	0.600
20.0	5	0.397	0.397
40.0	6	0.227	0.227

15.13.2.8.3 Using the equation from Section 4.2 in Appendix L, the estimate of the IC25 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j]\ \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC25 = 5.0 + [0.833(1 - 25/100) - 0.717]\ \frac{(10.0 - 5.0)}{(0.600 - 0.717)}$$

$$= 8.9 \text{ µg/L.}$$

15.13.2.8.4 Using the equation from Section 4.2 in Appendix L, the estimate of the IC50 is calculated as follows:

$$ICp = C_j + [M_1(1 - p/100) - M_j] \frac{(C_{(j+1)} - C_j)}{(M_{(j+1)} - M_j)}$$

$$IC50 = 10.0 + [0.833(1 - 50/100) - 0.600] \frac{(20.0 - 10.0)}{(0.397 - 0.600)}$$

$$= 19.0 \text{ µg/L.}$$

15.13.2.8.5 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC25 was 8.9286 µg/L. The empirical 95.0% confidence interval for the true mean was 3.3036 µg/L to 14.6025 µg/L. The computer program output for the IC25 for this data set is shown in Figure 6.

15.13.2.8.6 When the ICPIN program was used to analyze this set of data, requesting 80 resamples, the estimate of the IC50 was 19.0164 µg/L. The empirical 95.0% confidence interval for the true mean was 16.1083 µg/L to 23.6429 µg/L. The computer program output for the IC50 for this data set is shown in Figure 7.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	2.5	5.0	10.0	20.0	40.0
Response 1	.85	.81	.63	.63	.41	.12
Response 2	.78	.65	.74	.66	.41	.3
Response 3	.87	.71	.71	.51	.37	.2

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: sea urchin, Arbacia punctulata
Test Duration:
DATA FILE: urchin.icp
OUTPUT FILE: urchin.i25

Conc. ID	Number Replicates	Concentration µg/L	Response Means	Standard. Dev.	Pooled Response Means
1	3	0.000	0.833	0.047	0.833
2	3	2.500	.723	0.081	0.723
3	3	5.000	0.717	0.078	0.717
4	3	10.000	0.600	0.079	0.600
5	3	20.000	0.397	0.023	0.397
6	3	40.000	0.227	0.095	0.227

The Linear Interpolation Estimate: 8.9286 Entered P Value: 25

Number of Resamplings: 80
The Bootstrap Estimates Mean: 8.7092 Standard Deviation: 0.8973
Original Confidence Limits: Lower: 6.2500 Upper: 11.6304
Expanded Confidence Limits Lower: 3.3036 Upper: 14.6025
Resampling time in Seconds: 1.59 Random Seed: 1834854321

Figure 6. ICPIN program output for the IC25.

Conc. ID	1	2	3	4	5	6
Conc. Tested	0	2.5	5.0	10.0	20.0	40.0
Response 1	.85	.81	.63	.63	.41	.12
Response 2	.78	.65	.74	.66	.41	.3
Response 3	.87	.71	.78	.51	.37	.26

*** Inhibition Concentration Percentage Estimate ***
Toxicant/Effluent: Copper
Test Start Date: Test Ending Date:
Test Species: MYSID SHRIMP
Test Duration: fecundity
DATA FILE: mysidfe.icp
OUTPUT FILE: mysidfe.i50

Conc. ID	Number Replicates	Concentration µg/l	Response Means	Standard. Dev.	Pooled Response Means
1	8	0.000	0.934	0.127	0.934
2	7	50.000	0.426	0.142	0.426
3	7	100.000	0.317	0.257	0.317
4	8	210.000	0.000	0.000	0.000

The Linear Interpolation Estimate: 19.0164 Entered P Value: 50

Number of Resamplings: 80
The Bootstrap Estimates Mean: 19.0013 Standard Deviation: 0.8973
Original Confidence Limits: Lower: 17.6316 Upper: 21.2195
Expanded Confidence Limits: Lower: 16.1083 Upper: 23.6492
Resampling time in Seconds: 1.65 Random Seed: -823775279

Figure 7. ICPIN program output for the IC50.

15.14 **PRECISION AND ACCURACY**

15.14.1 PRECISION

15.14.1.1 Single-Laboratory Precision

15.14.1.1.1 Single-laboratory precision data for the reference toxicants, copper (Cu) and sodium dodecyl sulfate (SDS), tested in FORTY FATHOMS® artificial seawater, GP2 artificial seawater, and natural seawater are provided in Tables 13-18. The test results were similar in the three types of seawater. The IC25 and IC50 for the reference toxicants (copper and sodium dodecyl sulfate) are reported in Tables 13-16. The coefficient of variation, based on the IC25, is 28.7% to 54.6% for natural and FORTY FATHOMS® seawater, indicating acceptable precision. The IC50 ranges from 23.3% to 48.2%, showing acceptable precision.

15.14.1.2 Multilaboratory Precision

15.14.1.2.1 No data are available on the multilaboratory precision of the test.

15.14.2 ACCURACY

15.14.2.1 The accuracy of toxicity tests cannot be determined.

TABLE 13. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, OR OBTAINED DIRECTLY FROM NATURAL SOURCES, AND COPPER (CU) AS A REFERENCE TOXICANT[1,2,3,4,5]

Test Number	LOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	5.0	8.92	29.07
2	12.5	26.35	38.96
3	<6.2	11.30	23.93
4	6.2	34.28	61.75
5	12.5	36.67	75.14
n:	4	5	5
Mean:	NA	23.51	45.77
CV(%):	NA	54.60	47.87

1 Data from USEPA (1991a)

2 Tests performed by Dennis McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.

2 All tests were performed using FORTY FATHOMS® synthetic seawater.

3 Copper test solutions were prepared with copper sulfate. Copper concentrations in Test 1 were: 2.5, 5.0, 10.0, 20.0, and 40.0 µg/L. Copper concentrations in Tests 2-5 were: 6.25, 12.5, 25.0, 50.0, and 100.0 µg/L.

4 NOEC Range: < 5.0 - 12.5 µg/L (this represents a difference of one exposure concentrations).

5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 14. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN FORTY FATHOMS® ARTIFICIAL SEAWATER, OR OBTAINED DIRECTLY FROM NATURAL SOURCES, AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT[1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	<0.9	1.11	1.76
2	0.9	1.27	1.79
3	1.8	2.26	2.87
4	0.9	1.90	2.69
5	1.8	2.11	2.78
n:	4	5	5
Mean:	NA	1.73	2.38
CV(%):	NA	29.7	23.3

1 Data from USEPA (1991a)
2 Tests performed by Dennis M. McMullen, Technology Applications, Inc., EMSL, Cincinnati, OH.
3 All tests were performed using FORTY FATHOMS® synthetic seawater.
4 NOEC Range: <0.9 - 1.8 mg/L (this represents a difference of two exposure concentration).
5 SDS concentrations for all tests were: 0.9, 1.8, 3.6, 7.2, and 14.4 mg/L.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 15. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND COPPER (CU) SULFATE AS A REFERENCE TOXICANT [1,2,3,4,5,6]

Test Number	NOEC (µg/L)	IC25 (µg/L)	IC50 (µg/L)
1	12.2	14.2	18.4
2	12.2	32.4	50.8
3	24.4	30.3	46.3
4	<6.1	26.2	34.1
5	6.1	11.2	17.2
n:	4	5	5
Mean:	NA	22.8	29.9
CV(%):	NA	41.9	48.2

1 Data from USEPA (1991a)
2 Tests performed by Ray Walsh and Wendy Greene, ERL-N, USEPA, Narragansett, RI.
3 Copper concentrations were: 6.1, 12.2, 24.4, 48.7, and 97.4 µg/L.
4 NOEC Range: < 6.1 - 24.4 µg/L (this represents a difference of two exposure concentrations).
5 Adults collected in the field.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 16. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND SODIUM DODECYL SULFATE (SDS) AS A REFERENCE TOXICANT [1,2,3,4,5,6]

Test Number	NOEC (mg/L)	IC25 (mg/L)	IC50 (mg/L)
1	1.8	2.3	2.7
2	1.8	3.9	5.1
3	1.8	2.3	2.9
4	0.9	2.1	2.6
5	1.8	2.3	2.7
n:	5	5	5
Mean:	NA	2.58	3.2
CV(%):	NA	28.7	33.3

1 Data from USEPA (1991a).
2 Tests performed by Ray Walsh and Wendy Greene, ERL-N, USEPA, Narragansett, RI.
3 SDS concentrations were: 0.9, 1.8, 3.6, 7.3, and 14.5 mg/L.
4 NOEC Range: 0.9 - 1.8 mg/L (this represents a difference of one exposure concentration).
5 Adults collected in the field.
6 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 17. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN GP2, USING GAMETES FROM ADULTS MAINTAINED IN GP2 ARTIFICIAL SEAWATER AND COPPER (CU) SULFATE AND SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANTS[1,2,3,4,5]

	Cu (µg/L)				SDS (mg/L)			
Test	LC50	CI	NOEC	LOEC	LC50	CI	NOEC	LOEC
1	29.1	27.3-31.1	6.3	12.5	2.1	2.0-2.1	1.3	2.5
2	47.6	44.6-50.8	25.0	50.0	1.8	1.8-1.9	1.3	2.5
3	32.7	29.8-35.8	6.3	12.5	2.2	2.1-2.2	1.3	2.5
4	78.4	73.3-83.9	50.0	100.0	2.3	2.2-2.4	1.3	2.5
5	45.6	41.0-50.7	12.5	25.0	1.8	1.7-2.8	1.3	2.5
Mean	46.7				2.0			
SD	19.5				0.2			
CV	41.8				10.0			

1 Tests performed by Pamela Comeleo, Science Application International Corp., ERL-N, USEPA, Narragansett, RI.
2 All tests were performed using GP2 artificial seawater.
3 Copper concentrations were: 6.25, 12,5, 25.0, 50.0 and 100 µg/L.
4 SDS concentrations were: 0.6, 1.25, 2.5, 5.0, and l0.0 mg/L. SDS stock (14.645 mg/mL) provided by EMSL, USEPA, Cincinnati, OH.
5 For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.

TABLE 18. SINGLE-LABORATORY PRECISION OF THE SEA URCHIN, *ARBACIA PUNCTULATA*, FERTILIZATION TEST PERFORMED IN NATURAL SEAWATER, USING GAMETES FROM ADULTS MAINTAINED IN NATURAL SEAWATER AND COPPER (CU) SULFATE AND SODIUM DODECYL SULFATE (SDS) AS REFERENCE TOXICANTS[1,2,3,4]

	Cu (µg/L)				SDS (mg/L)			
Test	LC50	CI	NOEC	LOEC	LC50	CI	NOEC	LOEC
1	28.6	26.7-30.6	6.3	12.5	12.5	2.1-2.2	1.3	2.5
2	13.0	11.9-14.2	6.3	12.5	12.5	1.9-2.0	1.3	2.5
3	67.8	63.2-72.6	6.3	12.5	12.5	2.1-2.3	1.3	2.5
4	36.7	33.9-398	< 6.3	6.3	6.3	3.3-3.4	< 0.6	0.6
5	356	33.6-37.7	< 6.3	6.3	6.3	2.8-3.1	< 0.6	0.6
Mean	36.3				2.5			
SD	20.0				0.58			
CV	55.1				23.2			

[1] Tests performed by Anne Kuhn-Hines, Catherine Sheehan, Glen Modica, and Pamela Comeleo, Science Application International Corp., ERL-N, USEPA, Narragansett, RI.

[2] Copper concentrations were prepared with copper sulfate. Concentrations were 6.25, 12.5, 25.0, 50.0, and l00 µg/L.

[3] SDS concentrations were: 0.6, 1.25, 2.5, 5.0, and 10.0 mg/L. SDS stock (14.64 mg/mL) provided by EMSL, USEPA, Cincinnati, OH.

[4] For a discussion of the precision of data from chronic toxicity tests see Section 4, Quality Assurance.